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THE MECHANISM OF CHOLESTEROL ABSORPTION

DISSERTATION

SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY IN THE FACULTY OF PURE SCIENCE OF COLUMBIA UNIVERSITY

BY

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THE MECHANISM OF CHOLESTEROL ABSORPTION.

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By the analysis of chyle obtained from thoracic duct fistulas in dogs cholesterol which was fed has been shown[1] to be absorbed from the alimentary tract through the lymphatic system, whether fed as free cholesterol or as cholesterol esters. An increase in both fractions was always recognizable in the chyle, so that the normal proportion of one part of free to two or three parts of combined was quite constantly maintained. It seemed desirable to investigate further the mechanism of this process, particularly since, as has been pointed out,[2] there are rather important theoretical reasons for studying the process of a physiological interchange between free and combined cholesterol.

There are three general locations in which the esterification or saponification processes can take place during the absorption of cholesterol and its esters. *A priori* it might be expected from the general chemical and physical similarities between these bodies and the neutral fats that the same enzymes and tissues which are connected with the absorption of the latter must also take part in that of the former. The enzymes within the gastro-intestinal tract, the intestinal mucosa, and the mesenteric lymph nodes[3] all are concerned in the absorption of neutral fats. Cholesterol, during its absorption into the chyle of the thoracic duct, must be subjected to the activities of the same enzymes and tissues. The experiments to be described have been directed at all these agencies; and while it may be

[1] Mueller, J. H., *J. Biol. Chem.*, 1915, xxii, 1.
[2] Mueller, *J. Biol. Chem.*, 1916, xxv, 561.
[3] Stheeman, H. A., *Beitr. path. Anat. u. allg. Path.*, 1910, xlviii, 170.

stated here that the results do not furnish sufficient evidence for a complete explanation of the changes which apparently take place, yet it is thought they may throw some little light on the process.

Influence of the Gastro-Intestinal Tract on Cholesterol Absorption.

A possible change in the ingested cholesterol taking place in the stomach or intestine was sought for in three general ways: (1) by analysis of the gastric and intestinal contents after feeding cholesterol; (2) by following the absorption through a thoracic duct fistula with experimental elimination of certain of the digestive juices; and (3) by experiments *in vitro* dealing with the possible action of various enzymes upon cholesterol.

The determination of cholesterol was carried out by the Fraser and Gardner[4] modification of the Windaus[5] digitonin method. The extract of the material was prepared in the case of fluids, such as chyle, by the alcohol-ether method of Bloor,[6] and in the case of tissues, by treating the finely divided material with hot alcohol-ether followed by several extractives with boiling alcohol. To remove water-soluble extractives, which are always present, the combined extracts were evaporated to a small bulk, water and salt added (to prevent formation of emulsions), and the material extracted thoroughly with ether. The details of this method I have discussed elsewhere.[7]

Experiment 1.—After 24 hours' fasting, a dog was allowed to eat four raw eggs, shaken up in 350 cc. of water to which a little NaCl had been added. 10 cc. of this egg mixture were reserved for analysis. After 2 hours the stomach contents were removed by tube. A 20 cc. sample was analyzed. Peptic digestion had apparently been active, for the precipitate formed on adding alcohol to the digested sample was very light and fine compared to the heavy curdy precipitate of the fresh mixture.

[4] Fraser, M. T., and Gardner, J. A., *Proc. Roy. Soc., Series B,* 1910, lxxxii, 559.

[5] Windaus, A., *Z. physiol. Chem.,* 1910, lxv, 110.

[6] Bloor, W. R., *J. Biol. Chem.,* 1916, xxiv, 227.

[7] Mueller, *J. Biol. Chem.,* 1916, xxv, 549.

	gm		
Sample 1.			
Free	0.0236		
Combined	0.0020	C : T*	7.8
Sample 2.			
Free	0.0251		
Combined	0.0027	C : T	9.7

* C : T denotes the ratio of combined cholesterol to total cholesterol on a percentage basis.

Experiment 2.—A dog was allowed to eat a small piece of meat and three raw eggs. 4 hours later it was killed, and the contents of the small and large intestines were removed separately. The small intestine contained about 150 cc. of thick yellow fluid, and the large intestine about 30 cc. of material of the same appearance, together with some dark colored solid feces. The latter were discarded. About 10 cc. of each of the contents of the large and small intestines were analyzed.

	gm.		
Small intestine.			
Free	0.0369		
Combined	0.0016	C : T	4.2
Large intestine.			
Free	0.0227		
Combined	0.0022	C : T	8.8

As far as can be seen from these results, no esterification of free cholesterol appears to take place in the stomach or intestine. It is quite possible, however, in spite of such negative findings, that the ester might be formed slowly and be absorbed practically as fast as formed.

Experiment 3.—A dog was fed bread and milk containing 10 cc. of sesame oil in which 1.25 gm. of cholesterol oleate had been dissolved. After killing the animal 3 hours later, at which time the mesenteric lymphatics were white and prominent, the gastric and intestinal contents were analyzed. The stomach contained a considerable amount of undigested bread mixed with drops of oil. An unweighed portion of this was extracted and analyzed. Only about 5 cc. of a thick, bile-stained fluid could be obtained from the small intestine; this was extracted.

	gm.		
Stomach.			
Free	0.0139		
Combined	0.0256	F : T*	35
Intestine.			
Free	0.0360		
Combined	0.0077	F : T	82

* F: T denotes the ratio of free cholesterol to total cholesterol on a percentage basis.

These figures seem to indicate that hydrolysis of the ester takes place in the intestine. In view of experiments to be described later, however, this interpretation is not so probable, and it is well to note that if there were a selective absorption of the esters from the intestine, the result would be the same. Possibly only a part of the meal had reached the intestine, for while the whole of the intestinal contents was analyzed, the total cholesterol amounting only to some 0.04 gm., only a part, probably less than one-tenth, of the stomach contents was used, so that there was present in the stomach perhaps 0.5 gm. of cholesterol. Since a considerable part of the cholesterol present in the intestine must have come from the bile, evidently there was too little ingested cholesterol present to warrant any definite conclusions from this experiment.

By studying the absorption of cholesterol through the thoracic duct after experimentally diverting a single secretion, such as bile or pancreatic juice, from the intestine, it was hoped to gain an insight into the part played by such enzymes in the process.

Experiment 4.—Immediately after feeding a dog a small piece of meat, the animal was etherized, a thoracic duct fistula produced, and a sample of chyle collected. Then, through a laparotomy incision, 10 cc. of a solution of egg white, containing a small amount of dilute hydrochloric acid, were injected by means of a large hypodermic needle, directly into the duodenum. It was hoped in this way to start the normal digestive secretions into the intestine. This injection was followed by 10 cc. of cottonseed oil, containing 2 gm. of cholesterol oleate, given in the same way. The dog recovered promptly from the ether. After 2 hours chyle was obtained which was only moderately cloudy, and 200 cc. of milk were given the dog. In 2 hours more chyle which was very milky was collected and analyzed.

		Free.	Combined.	F : T.
		per cent	*per cent*	
11.00 a.m.	Thoracic duct fistula completed. Chyle collected................	0.022	0.038	36
11.30 "	Oil injected into intestine.			
1.30 p.m.	Chyle collected, moderately milky. Fed 200 cc. of milk.			
3.30 "	Chyle collected, very milky.			
5.30 "	" " " "	0.065	0.185	26

That gastric digestion is not an essential feature in the absorption of cholesterol is shown by the results of this experiment. Perfectly normal, if somewhat delayed, absorption took place when the solution of cholesterol in oil was injected directly into the duodenum. The delay probably resulted from a failure of the hydrochloric acid injection to bring about the pancreatic secretion, so that only after starting the normal sequence by feeding a little milk was the intestinal digestion properly started.

In the experiments now to be described, in which the bile was excluded from the intestine, the same general plan was followed in each case. The common bile duct of the dog was ligated, under ether, the gall bladder was brought up into the wound, a rubber tube, with a projection on its inner end formed by rolling it back upon itself, was fastened into the gall bladder by a purse string suture, and the wound was closed. Bile was removed three or four times a day through this cannula; at other times it was closed by means of a small clamp and protected by a close fitting oilcloth coat. In two instances the dogs became somewhat jaundiced on the following day, once due to hemorrhage into the gall bladder, with subsequent clotting and occlusion of the cystic duct; the cause for the other was not determined. Thoracic duct fistulas were made on the 2nd day after the biliary fistulas in order to give time for the bile present in the intestine to be eliminated as far as possible. Feces passed by the dogs during the course of the experiment were clay-colored, indicating the absence of bile.

Experiment 5.

		Free.	Combined.	F : T.
		per cent	*per cent*	
Dec. 30.	Biliary fistula made.			
Jan. 1.				
11.30 a.m.	Thoracic duct fistula completed. Chyle taken..................	0.021	0.028	43
1.30 p.m.	Chyle taken. Fed small piece of meat.			
1.45 "	Fed four raw eggs.			
5.00 "	Chyle taken, very milky.........	0.056	0.038	60
6.00 "	" " " "	0.058	0.037	61

Experiment 6.

Jan. 17.	Biliary fistula made.			
" 19.				
11.15 a.m.	Thoracic duct fistula completed. Chyle taken	0.020	0.037	35
5.15 p.m.	Fed three raw eggs.			
8.15 "	Chyle taken, very milky........	0.054	0.050	52
9.15 "	" " " "	0.050	0.037	57

Experiment 7.

Jan. 29.	Biliary fistula made.			
" 30.	Dog jaundiced (gall bladder contained clotted blood).			
Feb. 1.				
11.00 a.m.	Thoracic duct fistula completed. Chyle taken..................	0.037	0.044	46
12.00 "	Fed small piece of meat and three raw eggs.			
4.00 p.m.	Chyle taken, milky.............	0.051	0.063	45

Experiment 8.

Mar. 4.	Biliary fistula made.			
" 6.				
12.00 m.	Thoracic duct fistula completed.			
2.15 p.m.	Chyle taken. Fed four eggs.	0.022	0.045	33
5.15 "	Chyle collected, milky.	0.034	0.065	34
6.15 "	" " "	0.035	0.042	45

Experiment 9.

		Free.	Combined.	F : T.
		per cent	*per cent*	
June 6.	Biliary fistula made.			
" 8.				
11.30 a.m.	Thoracic fistula completed. Chyle taken..................	0.047	0.058	44
2.00 p.m.	Chyle taken. Given 200 cc. of milk and three eggs...........	0.045	0.058	44
5.30 "	Chyle taken, very milky........	0.054	0.060	47
8.15 "	" " " "	0.050	0.055	48

Experiment 10.

		Free.	Combined.	F : T.
June 10.	Biliary fistula made.			
" 12.				
10.00 a.m.	Thoracic duct fistula completed.			
2.30 p.m.	Given 200 cc. of milk. Chyle collected......................	0.057	0.094	38
	Fed 1.5 gm. of cholesterol oleate in 10 cc. of olive oil and 200 cc. of water.			
8.45 "	Chyle collected, quite milky..	0.073	0.087	46
	Fed 100 cc. of milk and then 1.5 gm. of cholesterol oleate in 10 cc. of cottonseed oil and 200 cc. of water, plus 3 gm. of dried ox bile.			
11.30 "	Chyle collected, quite milky.....	0.063	0.093	41

Experiment 11.

		Free.	Combined.	F : T.
June 12.	Biliary fistula made.			
" 14.				
10.00 a.m.	Thoracic duct fistula completed.			
1.00 p.m.	Chyle taken. Fed 1 gm. of cholesterol in 10 cc. of cottonseed oil and 200 cc. of milk....	0.038	0.066	36
5.00 "	Chyle taken. Fed 150 cc. of milk and then 1 gm. of cholesterol in 10 cc. of cottonseed oil and 200 cc. of water, plus 1 gm. of sodium taurocholate (Merck)..	0.041	0.065	39
9.00 "	Chyle taken, quite milky........	0.057	0.063	48
June 15.				
10.00 a.m.	" " " "	0.062	0.083	43

The bile apparently has a rather important function in connection with cholesterol absorption. Rothschild states[8] that after tying the common bile duct in dogs no cholesterol is absorbed. This evidently does not hold strictly. It each case there is a rise in the total cholesterol after feeding. This rise is small in some cases, and quite pronounced in others, but in no case is it so pronounced as that obtained in normal dogs. It does not depend upon the amount of fatty material absorbed, for in all cases, after feeding, chyle was obtained which was quite milky, the alcohol-ether extracts of which left a considerable amount of oily material after evaporation.

It has been suggested that bile aids the absorption of neutral fats by the reaction of the bile acids with the fatty acids, and in this way makes possible the entrance of the water-insoluble fatty substance into the cells of the mucosa. Cholesterol is also held in solution by bile acids, and its absorption may be facilitated in a similar manner.

The other noticeable fact brought out by the experiments is the relative increase in free cholesterol absorbed. In each case the percentage of free to total cholesterol was above that found normally after feeding. This figure seems to vary normally between perhaps 25 and 35 per cent, while in the biliary fistula animals it was between 39 and 61 per cent. The significance of this variation is not evident.

Normal absorption was not produced by the addition of bile or sodium taurocholate to the diet.

The experiments in which the pancreatic juice was excluded from the intestine were planned in the same way, allowing at least 2 days after the first operation for the dog to recover as far as possible. Under ether, the two pancreatic ducts were ligated and cut, and the pancreas itself, while left *in situ*, was separated from the intestine throughout its entire course, except for the larger blood vessels. In this way, it was believed, only the intestinal secretion would be eliminated, while the far reaching disturbances in carbohydrate metabolism, incident upon a complete removal of the pancreas, would be obviated. All the dogs recovered rapidly from the first operation, and were apparently in good condition when the thoracic duct fistulas were done.

[8] Rothschild, M. A., *Proc. N. Y. Path. Soc.*, 1914, xiv, 229.

Experiment 12.

		Free.	Combined.	F : T.
		per cent	*per cent*	
May 23.	Pancreatic ducts ligated and cut.			
" 27.				
11.00 a.m.	Thoracic duct fistula completed. Chyle taken..................	0.050	0.085	37.
2.00 p.m.	Chyle taken. Fed 200 cc. of milk and two eggs.			
4.30 "	Chyle taken....................	0.051	0.074	41
6.00 "	" "	0.058	0.077	43
8.00 "	" "	0.058	0.080	42

Experiment 13.

May 24.	Pancreatic ducts ligated and cut.			
" 27.				
12.00 m.	Thoracic duct fistula completed. Chyle taken..................	0.047	0.084	36
2.00 p.m.	Chyle taken. Fed 2 gm. of cholesterol oleate in 10 cc. of olive oil and the white of an egg in 150 cc. of water.			
4.30 "	Chyle taken, milky.............	0.047	0.075	39
6.00 "	" " "	0.053	0.077	41
8.30 "	" " "	0.057	0.074	43

Experiment 14.

May 29.	Pancreatic ducts ligated and cut.			
June 1.				
12.00 m.	Thoracic duct fistula completed. Chyle taken.			
2.45 p.m.	Chyle taken. Fed 150 cc. of milk and then 2 gm. of cholesterol oleate in 10 cc. of olive oil, in 150 cc. of water containing the white of an egg.	0.050	0.104	33
5.00 "	Chyle taken....................	0.045	0.082	36
8.15 "	" " quite milky........	0.057	0.083	41

Absence of the pancreatic juice seems to diminish the absorption of cholesterol very greatly. There is a slight increase in the free cholesterol after eating, but the cholesterol esters seem to decrease to some extent. The total cholesterol present remains nearly constant, but the ratio of free to total cholesterol rises somewhat. Here again, as in the case of the bile experiments, the decreased absorption cannot be due entirely to the absence of other lipoids in the chyle, for some of the samples at least were quite milky, although none contained as much fat as is present in normal digestion.

It seems evident that the pancreatic juice, and to a less extent the bile, exert an important influence on the absorption of cholesterol. If there could be demonstrated either a well marked hydrolytic action upon cholesterol esters, or an esterification of free cholesterol by one of the intestinal enzymes, it might help clear the situation. Experiments with this probability in view were undertaken with pancreatic extract, as well as with the intestinal mucosa of the dog. In the case of the pancreas, the organ was put through a meat chopper, and two or three parts of 50 per cent glycerol were added, to prevent as far as possible the activation of trypsin with the possible destruction of a lipase or "cholesterase." This suspension of hashed gland in dilute glycerol was used without filtering. The intestinal mucosa, after being scraped from the small intestine, was suspended in four parts of water. Since both cholesterol and cholesterol oleate are insoluble in aqueous media and solid at incubator temperature, a solution in sesame oil was used. This not only enabled the cholesterol to be presented to the enzyme action in a finely divided form, but also furnished an indication of ordinary lipase activity from the amount of fatty acid set free, which was estimated by titration.

For most of the experiments, four equal portions of 3 to 5 gm. of the ground pancreas or intestinal mucosa were taken, glycerol or water added to each, and then to each of one pair a small amount of sesame oil containing some free cholesterol, and to each of the other pair, some sesame oil containing cholesterol oleate, and, in some cases, 1 cc. of dog bile, were added. One of each pair was then mixed with alcohol-ether, and the

other, after the addition of a few drops of toluene, incubated from 1 to 6 days, with occasional shaking, and then extracted with alcohol-ether and boiling alcohol. The ether extract finally obtained was divided into two parts as usual, and the part intended for determination of total cholesterol was first titrated in alcohol solution with alcoholic KOH. In each case, as will be seen, there was quite a marked increase in fatty acids, an evidence of good lipase action. If the neutral fat-splitting enzymes could also split cholesterol esters, such action should be apparent here. On the other hand, with the gradual formation of fatty acids, there was abundant opportunity for esterification of the free cholesterol, if such a process could take place under these conditions.

Experiment 15. Pancreas plus Bile.—5 gm. portions of pancreas were used, and 0.4 cc. of sesame oil with, respectively, 0.01 and 0.013 gm. of cholesterol and cholesterol oleate. Incubated 24 hours.

	Titration.	Cholesterol.		F: T.
		Free.	Combined.	
	cc.	*gm.*	*gm.*	
Free cholesterol 0.01 gm., fresh........	3.0	0.0115	0.0007	94
" " 0.01 " incubated....	14.0	0.0042	0.0042	50
Cholesterol oleate 0.013 gm., fresh.....	3.9	0.0074	0.0047	61
" " 0.013 " incubated.	3.7	0.0015	0.0017	47

Experiment 16. Pancreas plus Bile.—5 gm. portions of pancreas were used, and to each of one pair was added 0.02 gm. of cholesterol in 0.8 cc. of sesame oil, and to the other about 0.04 gm. of cholesterol oleate in 1.0 cc. of sesame oil. Incubated 24 hours.

	Titration.	Cholesterol.		F: T.
		Free.	Combined.	
	cc.	*gm.*	*gm.*	
Free cholesterol 0.02 gm., fresh........	3.8	0.0212	0.0006	97
" " 0.02 " incubated...	14.8	0.0104	0.0102	50
Cholesterol oleate 0.04 gm., fresh......	3.5	0.0062	0.0058	52
" " 0.04 " incubated.	17.0	0.0032	0.0100	24

Experiment 17. Pancreas plus Bile.—4 gm. portions were used, and to each of one pair was added 0.02 gm. of cholesterol in 0.8 cc. of sesame oil, and to the other, 0.03 gm. of cholesterol oleate in 0.8 cc. of sesame oil. Incubated 6 days.

	Titration.	Cholesterol.		F: T.
		Free.	Combined.	
	cc.	*gm.*	*gm.*	
Free cholesterol 0.02 gm., fresh........	Lost.	0.0282	0.0008	97
" " 0.02 " incubated...	10.0	0.0165	0.0120	58
Cholesterol oleate 0.03 gm., fresh......	Lost.	0.0123	0.0136	47
" " 0.03 " incubated.	10.8	0.0090	0.0131	41

Experiment 18. Pancreas Alone.—4 gm. portions of pancreas were used. To each was added 0.02 gm. of cholesterol in 0.08 cc. of sesame oil. Incubated 24 hours.

	Titration.	Cholesterol.		F: T.
		Free.	Combined.	
	cc.	*gm.*	*gm.*	
Free cholesterol 0.02 gm., fresh........	4.6	0.0270	0.0000	100
" " 0.02 " incubated...	13.4	0.0210	0.0109	66

Experiment 19. Intestinal Mucosa plus Bile.—5 gm. portions were used. To each was added 1 cc. of dog bile, and to each of one pair, a solution of 0.02 gm. of cholesterol in 0.8 cc. of sesame oil, and to each of the other two, 0.03 gm. of cholesterol oleate in sesame oil. Incubated 24 hours.

	Titration.	Cholesterol.		F: T.
		Free.	Combined.	
	cc.	*gm.*	*gm.*	
Free cholesterol 0.02 gm., fresh........	1.0	0.0284	0.0022	93
" " 0.02 " incubated...	13.2	0.0270	0.0030	90
Cholesterol oleate 0.03 gm., fresh......	1.0	0.0136	0.0162	47
" " 0.03 " incubated.	11.4	0.0130	0.0142	48

In none of these experiments was there any indication of a hydrolytic effect on the cholesterol oleate, in spite of the lipase action which occurred. On the other hand, in all experiments in which the pancreas was used, a rather striking esterification

of the free cholesterol occurred. This was greatest in the experiments in which bile was used, but perfectly definite, also, in the fourth, in which there was only pancreas. In Experiment 1, the decreased amount of cholesterol found in the incubated samples is doubtless due to incomplete extraction. In the experiment with intestinal mucosa, there was no change demonstrable in either the free cholesterol or the oleate.

A synthetic action of this kind is not the rule for enzymes. True, a reversible effect can usually be demonstrated for most enzymes when conditions are carefully controlled. Here, however, the reaction apparently does not reach an equilibrium unless the greater part of the cholesterol has been esterified, since the samples to which cholesterol oleate had been added, and which contained only a relatively small amount of free cholesterol, also gave evidence of an esterification. On the other hand, since an enzyme is a catalytic agent which hastens a reaction that would take place slowly under ordinary conditions, it is not so unlikely that this should be a true enzyme action. Free cholesterol, as is well known, possesses strong inhibiting powers toward hemolytic substances of various kinds. It is not impossible that one of its functions is to combine with and render non-toxic, fatty acids which may become liberated in the body. Apparently it will combine very slowly with fatty acids set free in the brain after death.[9, 10] A raw egg mixed with water, and allowed to autolyze with tricresol and toluene for 6 months showed only 62 per cent of its total cholesterol to be free, as against about 90 per cent at the beginning. Seemingly there is a tendency for free cholesterol to unite slowly with fatty acids to form esters. It is quite conceivable that a body enzyme should hasten such a synthesis.

It is, of course, absolutely essential to show that this esterification did not take place during the extraction and analysis, from heating. The esters are prepared artificially by heating together the cholesterol and an excess of fatty acid to 200°. Possibly the evaporation on the water bath might have been enough to cause the change. Fortunately, the intestinal mucosa experiment, run parallel with those of the pancreas, acts as an

[9] Mair, W., *J. Path. and Bact.*, 1913–14, xviii, 179.

[10] Lapworth, A., and Royle, F. A., *J. Path. and Bact.*, 1914–15, xix, 474.

excellent control. In addition, the effect of evaporating a mixture of oil, fatty acid, and free cholesterol on the water bath was tried. About 0.01 gm. of cholesterol, 0.4 cc. of sesame oil, and 0.4 cc. of fatty acid from saponification of sesame oil were mixed with about 25 cc. of alcohol and 1 or 2 cc. of water, and evaporated down on a water bath. The process was then repeated and the residue allowed to heat for several minutes to 100° after the water and alcohol had evaporated. Analysis of this mixture and of a duplicate unheated mixture showed 0.0130 gm. of free cholesterol before heating, and 0.0131 gm. after heating. In other words, no esters had been formed.

As a further precaution to prevent any possibility of the esterification taking place from heating, the extraction of Experiments 3 and 4 was carried out with as little heating as possible, the extracts concentrated at room temperature before an electric fan, taken up in water, extracted by ether, the ether evaporated until the temperature rose to 45–50°, and then the fatty acids present accurately neutralized with alcoholic KOH, using phenolphthalein as indicator. The solution, after diluting with water, was again extracted with ether, and this solution, free from fatty acids, was analyzed as usual. No difference in results is apparent between these two experiments and the others.

From these experiments it seems fair to conclude that there is in the pancreas some substance which accelerates the formation of cholesterol esters and that possibly the bile increases this effect. It may well be that this action of the pancreas is of fundamental importance in the absorption of cholesterol and that here is to be found the explanation for the failure of cholesterol to be absorbed into the chyle after ligation of the pancreatic ducts.

Influence of the Intestinal Mucosa on Cholesterol Absorption.

In order to study the possible effect of the intestinal mucosa on the absorption of cholesterol, two methods were utilized. The first, an experiment *in vitro* with ground mucosa, already described, gave no evidence of any activity. The second was quite definite in its results. Portions of intestinal mucosa were analyzed during fasting of 24 hours' duration, and during the

active absorption of cholesterol or its esters. It was thought that with the rather considerable amount of fat present in the cells and lymph spaces of the mucosa, enough absorbed cholesterol might be present to be recognizable on analysis. In such experiments, digestion was allowed to proceed for 3 hours, and then the dogs were quickly killed by an intravenous injection of chloroform, the intestine was removed, opened, and washed as free as possible from adherent bile-stained mucus, and the mucosa scraped off with a dull knife. In the same way the material was obtained from dogs which had first been fasted for 24 hours.

The mucosa used for analysis was not carefully weighed out, and the figures are therefore given in grams of cholesterol found, rather than in percentage, and represent portions of mucosa of about 5 gm. weight.

	Duodenum.			Jejunum.			Colon.		
	Free.	Ester.	C: T.	Free.	Ester.	C: T.	Free.	Ester.	C: T.
	gm.	*gm.*		*gm.*	*gm.*		*gm.*	*gm.*	
24 hrs. fasting.....	0.0109*	None.	—						
24 " "	0.0092*	0.0006	6						
24 " "	0.0131	None.	—	0.0098	None.	—	0.0116	0.0006	4.9
Fed three eggs.....	0.0101	0.0021	17						
Fed 300 cc. of milk with 2 gm. of cholesterol in 8 cc. of cottonseed oil.	0.0111	0.0017	13						
Fed three eggs.....	0.0194	0.0061	24	0.0215	0.0064	23	0.0131	0.0014	9.7
Fed two eggs......	0.0079	0.0014	15	0.0105	0.0012	10	0.0071	0.0002	2.7

* A mixture of duodenum and jejunum mucosa analyzed.

In all the dogs which had been fasted, as well as in the mucosa of the large intestine of the others, there is comparatively little combined cholesterol, in some cases none, and in the others very little. On the other hand, the mucosa of the small intestine of all the dogs which had been fed showed considerably more cholesterol, the amounts ranging from 10 to 23 per cent of the total.

Evidently the cholesterol which is absorbed from the intestine has already been largely esterified by the time it reaches, or at least before it leaves, the lymphatics of the intestinal mucosa.

This fact of itself does not show whether the esters were formed in the intestinal canal and absorbed as such, or whether they were synthesized by the cells of the intestinal mucosa. Taken in connection with the work *in vitro* on the pancreas and intestinal mucosa, however, it seems very probable that the change takes place slowly in the intestine, and that an absorption of the esters takes place as rapidly as they are formed.

No striking difference in the direction of a higher percentage of free cholesterol is apparent in these experiments in which cholesterol was fed. Such an increase, if it existed, could not be recognized with any certainty even by the average results of a considerable number of experiments, for it evidently could not be very great, and there seems to be considerable individual variation in the normal cholesterol content of the intestinal mucosa.

Influence of the Mesenteric Lymph Nodes on Cholesterol Absorption.

No satisfactory experiment could be devised to show a possible effect of the mesenteric lymph nodes on the absorbed cholesterol. It was first attempted to destroy the lymphoid tissue of the body by massive doses of x-ray. A rather small dog was exposed to the rays for about half an hour each day for 5 successive days, during which time the small lymphocytes in the blood fell from 26 to 3 per cent.[11] A thoracic duct fistula was then made, and an analysis of the chyle obtained before and after feeding eggs showed a perfectly normal absorption. After completing the experiment the dog was killed, and the mesenteric lymph nodes, the thymus, and the spleen were examined. The thymus was extremely atrophic, although the dog was quite young. The spleen was rather small and firm, but lymphoid follicles were visible in the gross. The lymph nodes were normal in the gross, but microscopically the germinal centers were somewhat small, and the pulp seemed to contain much less than the usual number of lymphoid cells. But the destruction was by no means sufficient to make it unlikely that they would exercise a normal function on the fat and lipoids passing through them in the chyle.

An attempt to demonstrate the presence of an enzyme in the gland by a technique similar to that used in the case of the pan-

[11] I am indebted to Dr. M. J. Sittenfield for raying the animal for me.

creas and the intestinal mucosa also gave negative results. Fatty acids were produced by the lipase of the glands, and the experiments may thus also serve as controls on the earlier experiments with pancreas.

	Titration.	Free.	Combined.	F: T.
	cc.	*gm.*	*gm.*	
Free cholesterol, fresh	0.9	0.0163	0.0002	99
" " incubated	5.0	0.0136	0.0006	96
Cholesterol oleate, fresh	0.5	0.0047	0.0037	56
" " incubated	1.3	0.0036	0.0028	56

In none of the experiments is there any indication either of a saponification of the esters, or of an esterification of the free cholesterol. The lower values found in the fourth are doubtless due to incomplete extraction, for here again it was attempted to avoid an accidental esterification of cholesterol through heating with the fatty acids formed, by the procedure previously described of extracting with no more heat than necessary, and subsequently evaporating before an electric fan, instead of distilling, and neutralizing the fatty acids present.

DISCUSSION.

Of the experimental data here presented there are only a few facts which stand out as definite. First, the bile, and even to a greater extent the pancreatic secretions, seem intimately connected with cholesterol absorption, indeed to a degree somewhat out of proportion to their influence on neutral fat absorption. By experiments *in vitro* it was shown that free cholesterol, in the presence of fatty acids and a suspension of pancreas, undergoes esterification, and from control experiments it seems most likely that this is a real action by the pancreas. In such experiments the addition of a little bile seems to make the change somewhat greater, but not enough experiments of this kind were done to make any generalizations.

By analysis of the intestinal mucosa during starvation and after feeding it was shown that an esterification of at least a large part of the absorbed cholesterol had already taken place.

Since no effect of the mucosa could be demonstrated *in vitro*, it is at least possible that the esterification may take place in the lumen of the intestine under the influence of the pancreatic juice, and that the esters may be absorbed as rapidly as formed.

Again, no evidence that the mesenteric lymph nodes take any part in cholesterol absorption could be obtained. The evidence of their part in neutral fat absorption is, as far as I am aware, based on morphology and differential fat staining, although lymphoid tissue undoubtedly contains an active lipase.

One fact which stands out prominently is the resistance of cholesterol esters to saponification by ordinary active lipases. In no case has there been the slightest evidence of splitting, although neutral fat in the same mixture was easily saponified.

Taken altogether the data here presented by no means explain the whole process of cholesterol absorption. The peculiar effect of the bile in altering the proportion of free cholesterol absorbed is entirely unexplained. More important yet, while the origin of the cholesterol esters seen in the chyle of normal animals after feeding cholesterol-rich meals has been at least in part explained, the increase in free cholesterol and the mechanism which regulates the proportion of the two and keeps it constant has not been cleared up at all. However, the inherent difficulties encountered in carrying out the work make it appear quite useless to seek a more complete understanding of the process by means of the methods at present available.

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He is the author of the following publications:

(With Charles J. Robinson) An Improved Technic for the Doremus-Hinds Ureometer. *J. Am. Med. Assn.*, 1914, lxii, 514.

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